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New *in Vitro* Findings on the "Free" Form of Apolipoprotein A-1

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INTRODUCTION

Investigations on lipoproteins are extremely relevant to aging research because of the well-known occurrence in elderly people of lipoprotein disorders, atherosclerosis, and coronary heart disease (CHD). Several epidemiological studies have established an inverse relationship between CHD and the concentration of high-density lipoproteins-cholesterol (HDL-cholesterol) in serum.[1] High-density lipoproteins play an important role in reverse cholesterol transport from peripheral cells back to the liver.[2] Plasma solubility and transport of HDL are mediated by apolipoprotein A-I (apo A-1), a molecule that also contributes to the structure of the HDL particle, to its interaction with cell receptors, and finally to lipoprotein metabolism inside the cell, where apo A-I acts as a regulator of enzyme activities. A decade of research on apolipoproteins established that the protein components of lipoproteins seem to be better predictors than lipoproteins in the assessment of the risk of atherosclerosis,[3-5] and that apo A-I concentration is inversely related to the risk of CHD. In the clinical laboratory apo A-I is studied by immunoassay methods such as immunenephelometry (IN), radial immunodiffusion (RID), and electroimmunoassay (EIA). It is of great importance for the clinical laboratory to develop appropriate methods for the quantification of serum HDL pool size and apo A-I determination, and the detection of an important component present in human serum—namely, the (non–HDL-associated) so-called "free" apolipoprotein A-1. Besides describing new methods for investigating free apo A-I in serum and HDL-cholesterol surnatants, this paper also gives preliminary data on *in vitro* behavior and *in vivo* variations in some pathophysiological conditions. Finally, the paper describes the differential reactivity of free apo A-I with polyclonal and monoclonal antibodies, a finding that may introduce variability in some immunoassay methods used for the assessment of atherosclerosis risk.

MATERIALS AND METHODS

Immunofixation electrophoresis (IFE) combines agarose gel electrophoresis and immunoprecipitation.[6] The detailed procedures of its application to the detection and quantitation of apolipoproteins have previously been described by our group.[7] Peripheral venous blood was sampled from the subjects tested after an overnight fast; after coagulation and centrifugation, the sera were collected and electrophoresed on agarose gel plates (Sebia, Issy-les-Moulineaux, France; Beck-

man, Brea, CA). All the experiments were carried out on fresh, undiluted sera unless otherwise indicated.

HDL-cholesterol surnatants were prepared according to the conventional procedure, utilizing the dextran-sulfate/$MnCl_2$ precipitation procedure.

Antisera. The polyclonal monospecific anti-apolipoprotein A-1 antibodies were from Beckman (Brea, CA), Behring (Scoppito, AQ, Italy), and Boehringer Mannheim (Milan, Italy). Anti-human apolipoprotein A-1 murine monoclonal antibodies used were: clones A/11 (isotype IgG.K), 253/8 (isotype IgG1.K), 43/1 (isotype IgA.K), 95/2 (isotype IgG1), A/13 (isotype IgG1.K); all were obtained from Chemicon (Diagnostic Brokers Associated, Milan, Italy).

Radial immunodiffusion was performed in ready-to-use agarose gel plates containing anti-human apolipoprotein A-1 murine monoclonal antibody mixture from Behring (Scoppito, AQ, Italy).

FIGURE 1. The IFE pattern obtained with two normolipidemic sera after immunoprecipitation with anti-human apo A-1 polyclonal antiserum. The *lower minor bands* correspond to free apo A-1. The *upper major bands* correspond to alpha(HDL)-associated apo A-1.

Ouchterlony-coupled RID (OCRID) is a new method, devised by one of the authors (A.S.), and herein described for the first time. OCRID associates two different immunological procedures within a single assay; indeed, it couples RID with double-immunodiffusion, thus allowing the detection of those immunoreactivities not neutralized during the radial immunoprecipitation, which are then detected as precipitin bands (see RESULTS).

RESULTS

The free form of apolipoprotein A-1 (free apo A-1) corresponds to the lower minor bands visible in the IFE shown in FIGURE 1; the upper major bands corre-

FIGURE 2. The IEF pattern b-tained with four different human normolipidemic sera challenged with anti-human apo A-1 polyclonal antiserum. Free apo A-1 appears as a *tail* at the bottom of an upper major precipitin arc (HDL-apo A-1). The **right half** of the figure shows the same IEF as the left, but stained for lipids, resulting in the disappearance of the free apo A-1 *tails*.

spond to the alpha(HDL)-associated apo A-1. The IFE shown in FIGURE 1 is stained for proteins; if we stain the same figure for lipids (data not shown), the free apo A-1 bands almost disappear, suggesting a very low lipid content.

The overall picture described above for IFE can also be detected by the conventional immunoelectrophoresis (IEF) (see FIG. 2), where free apo A-1 appears as a tail at the bottom of a major precipitin are corresponding to the alpha(HDL)-associated apo A-1 immunoreactivity. The right part of the figure shows that if we stain the same IFE for lipids, the lower cathodic precipitin tail almost disappears, leaving the upper alpha-associated arc.

The same pattern of IFE shown in FIGURE 1 and of IEF shown in FIGURE 2 is detectable in HDL-cholesterol surnatants after polyanionic treatment of sera followed by centrifugation (data not shown).

The laboratory probing for free apo A-1 requires the use of freshly collected sera; indeed, if normal human sera once collected are maintained for 5 days at +4°C, then upon IFE execution, a clear-cut increase of free apo A-1 appears (see FIG. 3), suggesting a displacement of the apolipoprotein moiety from HDL.

FIGURE 3. When IFE is performed on *in vitro* aged sera (not freshly collected), a clear-cut increase in free apo A-1 appears. The two sera are from normolipidemic donors.

As far as the behavior of free ap A-1 in pathological conditions is concerned, besides the previously reported[7,8] increase of free apo A-1 in some forms of hypertriglyceridemia, we herein report for the first time preliminary data on a few patients with liver cirrhosis who show a decrease of free apo A-1 up to complete disappearance (see FIG. 4).

To return to *in vitro* findings, a major observation made during this investigation was the demonstration of the failure of anti-human apo A-1 murine monoclonal antibodies and monoclonal antibody mixture to immunoprecipitate free apo A-1 both in IFE (see FIG. 5) and IEF (see FIG. 6). Both figures clearly show that, when a normal human serum, previously shown to possess a free apo A-1 immunoreactivity, is challenged with monoclonal antibodies, only the alpha(HDL)-associated apo A-1 is immunoprecipitated. FIGURE 5 also shows a prozone effect due to antigen excess. This finding may be important because several laboratories worldwide

FIGURE 4. Decrease to the point disappearance of free apo A-1 in the sera of two patients with liver cirrhosis.

utilize commercially available RID assays using monoclonal antibody mixture. Therefore, with the aim of evaluating whether if all (total)apo A-1 immunoreactivity was immunoprecipitated in a RID assay working with monoclonal antibody mixture (Behring Institute), we devised (see MATERIALS AND METHODS) a new technique that combines radial immunodiffusion on ready-to-use plates with double immunodiffusion (Ouchterlony). This aim was pursued by introducing a new well in the agarose and filling it with 5 μl of anti-apo A-1 monoclonal antiserum near the normal RID well containing the patient serum. A clear-cut precipitin line appeared between the two wells (see FIG. 7), suggesting that an apo A-1 immunoreactivity escaped immunoprecipitation by the monoclonal antibody mixture during the radial immunodiffusion. As far as we know, this is a new, previously unreported finding obtained with a new, previously unreported technique, which we call Ouchterlony-coupled RID (OCRID). The overall data obtained with three different

FIGURE 5. Anti-human apo A-1 monoclonal antibody mixtures fail to precipitate free apo A-1, but precipitate HDL-associated apo A-1, giving a prozone effect due to antigen excess.

methodological approaches (IFE, IEF, and OCRID) suggest that monoclonal antibodies directed against human apo A-1, while succeeding in precipitating the HDL-linked form of apolipoprotein A-1, fail to immunoprecipitate the HDL-free form.

DISCUSSION

Although the atherogenic role of cholesterol has been under investigation since the early years of this century, the exact pathogenetic connections between atherosclerosis and cholesterol still need research efforts. Further investigation is also needed for the role of HDL-cholesterol in preventing/protecting against or at least delaying atherosclerosis and its sequelae. Taking into account that apolipoprotein

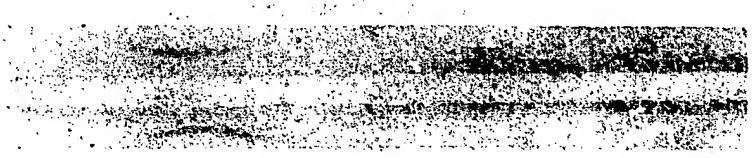

FIGURE 6. The IEF pattern obtained when a normal human serum is challenged with anti-human apo A-1 monoclonal antibody mixture. Only HDL-associated apo A-1 gives a precipitin arc; free apo A-1 is not immunoprecipitated.

A-1 has now emerged as an important parameter inversely related to risk CHD, new laboratory approaches for investigating this apolipoprotein seem to be important for a deeper insight into pathophysiological conditions that are relevant to the elderly.

Besides lipoprotein HDL-linked apo A-1, the presence in human serum of a form of apo A-1 non–HDL-associated that has been called "free" apo A-1 has now been well established.[9] Free apo A-1 should not be confused with pure apo A-1, which is an artificial laboratory preparation of the molecule in a lipid-free state. An apo A-1 component migrating cathodally to HDL in immunoelectrophoresis was first described by Levy and Fredrickson.[10] Thereafter, this form of apo A-1 has been called "free" apo A-1.[9] So far, methods such as ultracentrifugation,[11] and crossed-immunoelectrophoresis[12] were necessary for determining the presence of this singular apolipoprotein. However, these techniques were not adequate or simple enough for routine use in the clinical laboratory, and they were therefore restricted to research laboratories having the necessary equipment or special

FIGURE 7. Ouchterlony-coupled RID: monoclonal antibody mixture (anti-human apo A-1) fails to precipitate all the apo A-1 immunoreactivity during RID. A precipitin line results in the following double-immunodiffusion with an anti-apo A-1 polyclonal monospecific antiserum. The RID plate has been stained for proteins.

competence. It has been known for many years that plasma proteins can be precipitated after electrophoresic separation by the use of specific antisera.[6] Recently, we demonstrated that such precipitation can detect apolipoproteins in agarose gel after the preliminary separation of lipoproteins by electrophoresis.[7] On the basis of these findings, we have herein focused our attention on the great potential of IFE as a method for detecting and studying the free form of apolipoprotein A-1. The important advantage of this new approach is its simplicity and reliability. The quantitative determination of this component within total apo A-1 is also possible by means of densitometric scanning, and this approach is now under optimization and refinement in our laboratory.

Besides its technical aspects, in this paper we also report new findings on free apo A-1: its marked increase in *in vitro* aged sera, its decrease to the point of disappearance in some subjects with liver cirrhosis, and finally the failure of monoclonal antibodies to cause its precipitation in agarose gel. Indeed anti-apo A-1 monoclonal antibodies, while succeeding in precipitating HDL-associated apo

A-1, fail to precipitate the free form; this is an important new finding both from the point of view of basic knowledge of this protein and for its practical implications concerning immunoassay determinations using monoclonal antibodies in RID assays. Taking into account that free apo A-1 normally constitutes about 10% of total apo A-1 and can reach higher concentrations in some hypertriglyceridemic patients, it might constitute a major source of variability also within the same immunoassay (RID) because of the differential utilization of polyclonal monospecific antisera versus monoclonal antibody mixtures. We are confident that the new techniques here used, IFE and OCRID (the latter here described for the first time), as well as the new findings herein reported, can contribute to and suggest new insights into the as-yet-unknown pathophysiological significance of the free form of apolipoprotein A-1, particularly in dislipidemic conditions, CHD, and aging.

SUMMARY

Clinical and experimental gerontologists are extremely interested in lipoproteins as well as in new methods for investigating and probing the apolipoprotein pattern. Using immunofixation electrophoresis, we separated free apolipoprotein A-1 from the apo A-I associated with high-density lipoproteins. Free apolipoprotein A-1 is a low-molecular-mass form of apo A-1 that seems to contain an extremely low quantity of lipids. The use of IFE as a tool for probing free apo A-1 has revealed new and interesting findings, such as its "artificial" increase during serum conservation at temperatures between 0–4°C. From the clinical point of view, we demonstrated a decrease to the point of disappearance of free apo A-1 in some patients with liver cirrhosis. Moreover, one of the main findings here reported is the failure of anti-human apo A-1 murine monoclonal antibody and monoclonal antibody mixture to precipitate free apo A-1 in agarose systems. This discovery has important implications both for basic knowledge on apolipoproteins and for practical reasons concerning variability in those immunoassays (radial immunodiffusion) utilizing monoclonal antibody mixtures.

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